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INHIBITORY EFFECT OF PREMNA TOMENTOSA (L.VERBENACEAE) ON STELLATE CELL ACTIVATION AND COLLAGEN ACCUMULATION IN DMN INDUCED HEPATIFIBROSIS IN RATS.

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ABSTRACT

Premna tomentosa is a key medicinal plant widely used in Indian ayurvedic medicine for the treatment of liver diseases. The beneficial effects are due to its antioxidant and anti-inflammation action. We have studied the protective action of Premna tomentosa against activation of liver stellate cells and accumulation of collagen and *hyaluronic acid* during DMN induced hepatic fibrosis in rats. The expression of *hyaluronic acid* and collagen accumulation were considerably reduced during Premna tomentosa treatment. Similarly, a significant decrease was observed in elevated levels of serum transaminases and lipid peroxide in Premna tomentosa treated animals when compared with DMN administered group the results suggest the protective role of Premna tomentosa treated animals when compared with DMN administered group. The results suggest the protective role of Premna tomentosa against activation of liver stellate cells and accumulation of connective tissue proteins during DMN induced fibrosis in rats. Premna tomentosa may be used as an effective antifibrotic agent for drug induced liver damages.

Key words: Hepatic fibrosis, Hepatic stellate cells, DMN, Premna tomentosa

INTRODUCTION

Hepatic fibrosis has been a common response to chronic liver injury and might result in potentially lethal sequelae (1, 2). Liver diseases in general are caused by various factors such as persistent viral hepatitis, alcohol abuse, chronic liver diseases, heavy metal toxicity and environmental toxins (3). It is cardinal to treat liver disorders since it directly affects the biochemistry of the cell through participating events like oxidative stress, redox change etc., (4).

Lipid peroxidation results in structural and functional integrity of cell membranes, which also alter the activities of various membrane bound ATPases (5, 6). It has been demonstrated that DMN induced liver injury in rats is a good and reproducible animal model for studying biochemical and path physiological alterations with the development of hepatic fibrosis and cirrhosis (7, 8, 9, 10). It has been shown that lipid peroxidation is associated with hepatic fibrosis and stellate cell activation.

In recent years much importance is emphasized on natural products in diet and usage of more medicinal plants in

pharmaceuticals due to naturally occurring active principles with fewer side effects. Premna tomentosa L. (Verbanacae), commonly called as 'Podaganari' and 'Krishnapalai' is a medicinal plant used extensively for the treatment of various disorders. It is a moderate sized deciduous tree with shoots, leaves and inflorescence densely clothed with a tawny yellow stellate tomentum. Bark is light grayish brown, like that of teak and flowers are greenish yellow. Premna tomentosa is a medicinal plant widely used in Indian ayurvedic medicine for the treatment of liver diseases. (11). Earlier studies have shown that the methanolic extract of Premna tomentosa leaves afforded significant protection against acetaminophen-induced hepatoxicity in rats by its antioxidant property in rats (12). The preliminary work was presented in Conferences (13). In the present study we have shown the inhibitory effect of Premna tomentosa (L.Verbenaceae) on stellate cell activation and collagen accumulation in DMN induced hepatic fibrosis in rats

MATERIALS AND METHODS

The plant material was collected and authenticated by Dr. Saradha Vasanth, Central Research Institute for sidhha, Chennai, India. The powered leaves were extracted with methanold to a syrupy mass. The last trace of the solvent was removed in vacuum with an appropriate yield of 12.4%. The method of Gilani and Janbaz, 1995 (14) was followed for induction of acute hepatotoxicity

Experimental Procedure

Male albino rats of Wistar strain, weighing approximately 150-200 g, were obtained from the Tamilnadu University of Veterinary and Animal Sciences, Madhavaram, Chennai, India. The animals were housed in solid-bottomed polypropylene cages and received commercial rat chow (Hindustan Lever Ltd, Bangalore, India) and water, ad libitum. The animals were divided into four groups with six rats in each group.

Group 1 - Control animals received normal rat diet and water, ad libitum.

Group 2 - Drug control, received normal rat diet and were given Premna tomentosa (0.5 ml/100 g.b.wt.), orally for 30 days.

Group 3 - Hepatic fibrosis was induced by intraperitoneal injections of Dimethylnitrosamine in doses of 1mg (prepared in 0.15M NaCl)/100g body weight for 7 consecutive days.

Group 4 - Premna tomentosa + DMN (Same dosage as above)

Biochemical parameters

All the animals were sacrificed 72h after the last administration of DMN, Premna tomentosa. Treated and control animals were anesthetized with diethyl ether, and the blood was collected by cutting the right jugular vein on the neck. Na^+ K^+ ATPase and Ca^{++}ATPase activities were measured in isolated plasma membranes according to the procedure of Bonting (15) and Hjerten and Pan (16) respectively. Lipid peroxides were also measured in liver homogenates according to the method of Ohkawa et al (17). Liver collagen content was estimated by measuring hydroxyproline by the method of Woesnner (18).

Isolation and Purification of Ito Cells from Rat Liver

The procedure for isolation of rat hepatic cells (Ito Cells) was followed according to the procedure of Knook et al (19) by portal vein perfusion method. Briefly, hepatic stellate cells were isolated by pronase-collagenase digestion and were activated by culture on uncoated plastic. The resulting cell suspension was filtered through sterile gauze and centrifuged at 50 x g for 3 min to remove hepatocytes and debris. The suspension after removal of sediments containing non-parenchymal cells were layered over 25% preformed (20,000 x g for 10 min) percol gradient and centrifuged at 800 x g for 30 min. The gradient centrifugation produced a top layer of yellowish white oily debris with a band of cells immediately below.

The band mainly contained stellate cells. These cells were identified by autofluorescence at 325nm that rapidly faded under fluorescent microscope and exhibited round morphology. Staining for desmin was used as the confirmatory marker. The cells also displayed > 95% viability following trypan blue exclusion protocol.

Cell culture

The freshly isolated hepatic stellate cells were washed thoroughly in Dulbecco's Minimal Essential Medium and plated in DMEM supplemented with 10% FBS and plated at a density of 1.0 x 106 cells per 100 mm tissue culture dish in DMEM supplemented with 10% FBS. The cells were maintained at 37°C under humidified 5% CO2 - 95% air on a 100 mm tissue culture dish. The medium was changed every 24 hrs. As the cells reached confluence, they were passaged at a 1:4 split ratio after they were removed from the dish with a brief exposure to 0.25% trypsin - 0.02% EDTA in phosphate buffered saline (PBS).

Microscopic observation

Cultured cells were viewed with a WILOVERT inverted microscope and photographed using KODAK Ektachrome film. Immunostaining of a - smooth muscle actin (α–SMA) was done according to the procedure of Buchwalow et al (20).

Statistical analysis

Arithmetic mean and standard deviations were calculated data. The results were statistically evaluated using one-way analysis of variance (ANOVA). The control mean values were compared with the treated mean values by the least significant difference method. The value of $P<0.05$ was considered as statistically significant.

RESULTS

Table 1 shows the Levels of Liver collagen, MDA, Hyalunoic acid, Na^+ K^+ ATPase, Ca^{++} ATPase activity in control and Experimental groups. A significant increase in liver collagen content of 37.4 ± 7.9 mg/mg protein was observed in DMN treated rats when compared to the untreated controls. A notable reduction of collagen level in DMN + Premna tomentosa treated groups. Increased MDA levels during DMN induction were observed. Treatment with Premna tomentosa groups showed lesser MDA levels, which could be attributed to preventive effect of Premna tomentosa in hepatic fibrosis formation. DMN treated group exhibits increased activity of Na^+ K^+ ATPase and Ca^{++} ATPase when compared to control groups. Premna tomentosa prevented the increase of ATPase. During DMN induction in group 3 there was significant elevation in collagen MDA and hyaluronic acids. Group-3 showed lesser collagen and hyaluronic acid while MDA levels were significantly reduced. Group 2 drug control shows near normal values comparable with control groups.

Fig 1 shows Hematoxylin and Eosin Staining of Rat Liver After DMN Administration Demonstrating Necrosis. Control Liver shows the normal architecture with central vein and radiating cords, Several foci of spotted necrosis was observed in day 3. Extensive necrosis and hemorrhage with marked neutrophilic and mononuclear infiltration during day 5. Day 7 and 14 Massive hepatic necrosis and collapse of liver parenchyma and bridging necrosis were observed. Day 21 the Intensive fibrosis with thick collagen fibers was shown. Figure 2 shows the Masson's Trichome staining of experimental rat liver. Control Liver shows the normal Architecture with central vein and radiating cords, 3 day of staining shows the Slight percentile fibrosis, Bridging fibrosis with initiation of collagen fibers was observed in Day 5. During 7th day Liver specimen demonstrating well developed fibrosis and Day 14 shows focal fibrosis and clear cirrhosis and finally Day 21 Delineated cirrhosis.

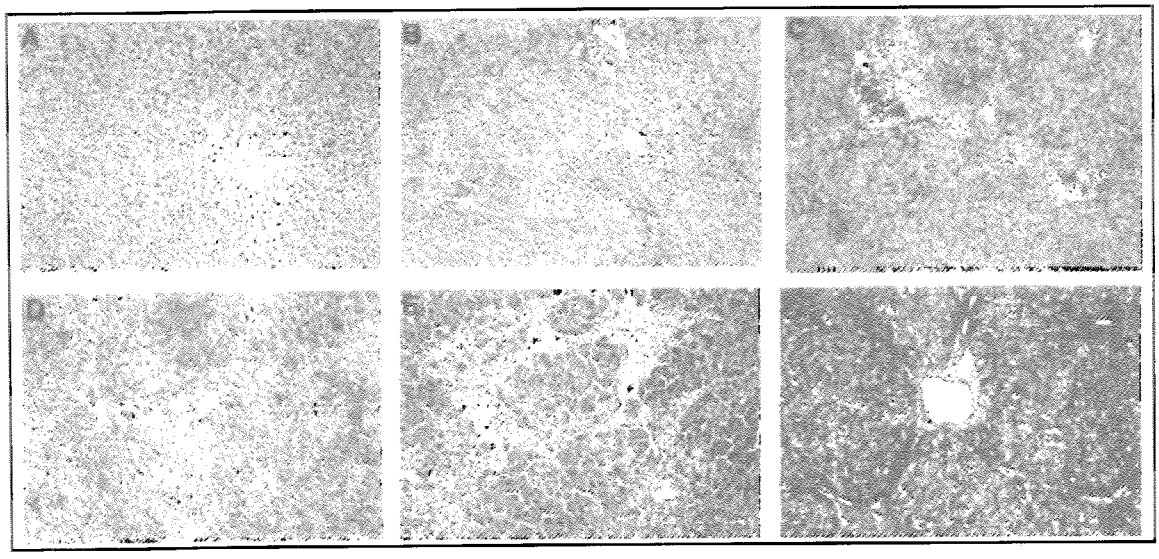
Fig 3 shows Staining Of Alpha Smooth Muscle Actin. Control Liver shows Staining of alpha smooth muscle actin absent totally. Day 3 shows prominent number of stellate cells become activated and stained for alpha smooth muscle actin and the number of alpha smooth muscle actin stained stellate cells increased

Table 1: Levels of Liver collagen, MDA, Hyaluronic acid, Na+ K + ATPase, Ca2+ ATPase activity in control and Experimental groups

Parameters	Group 1	Group 2	Group 3	Group 3
Liver collagen (μg/mg protein)	14.9 ± 4.9	37.4 ± 7.9 *	26.9 ± 3.9 *	14.3 ± 4.3 #
MDA (nmoles of malondialdehyde/mg protein)	0.23 ± 0.08	0.8 ± 0.7 *	0.6 ± 0.05 *	0.19 ± 0.07 #
Hyaluronic acid (ng/mg protein)	68.3 ± 15.3	32.0 ± 56.4 *	108.7 ± 13.8 *	70.3 ± 13.9 #
$Na^{+}K^{+}$ATPase (μmoles Pi/mg.h)	13.8 ± 9.4	5.9 ± 2.2 *	8.1 ± 4.3 *	12.8 ± 5.6 #
Ca^{++}ATPase (μmoles Pi/mg.h)	1.5 ± 0.5	0.2 ± 0.7 *	1.4 ± 0.9 *	1.5 ± 0.7 #

Values are expressed as mean ± SD
P <0.05, * Compared with Control group; # Compared with Control and DMN treated group

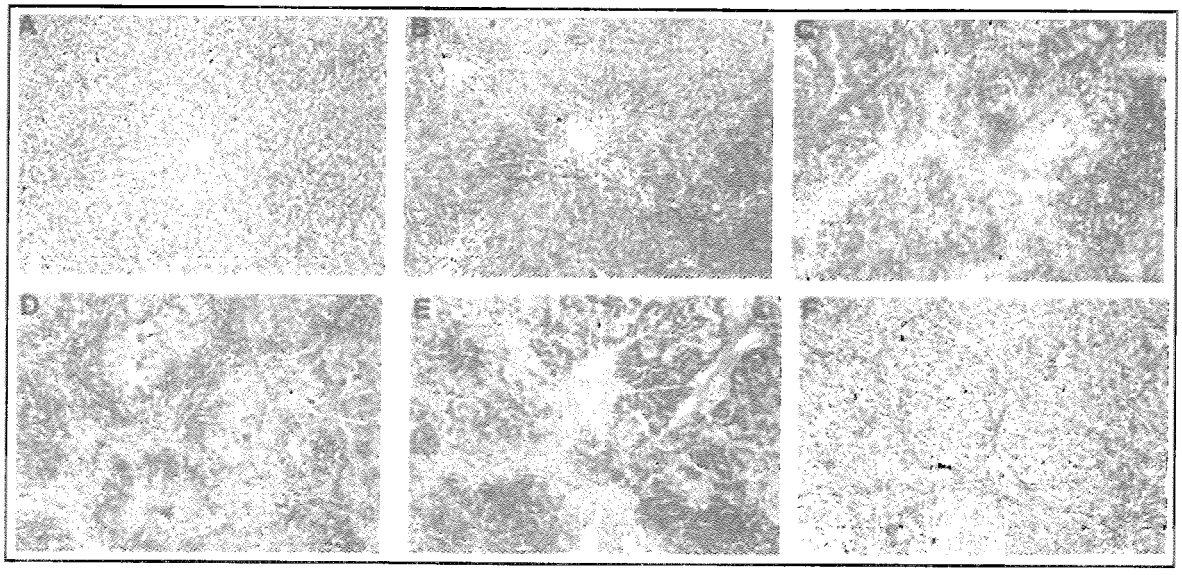
Fig 1: Hematoxylin and Eosin Staining of Rat Liver After DMN Administration Demonstrating Necrosis



A Control Liver: Normal Architecture with central vein and radiating cords
B Day 3: Several foci of spotted necrosis
C Day 5: Extensive necrosis and hemorrhage with marked neutrophilic and mononuclear infiltration
D Day 7: Massive hepatic necrosis and collapse of liver parenchyma
E Day 14: Bridging necrosis was observed
F Day 21: Intensive fibrosis with thick collagen fibers

during day 5. During Day 7 the Density of positive stained stellate cells in necrotic zone was high. Staining was restricted to fibrotic zone in day 14. Finally the 21st day the Density of positive stained cells in necrotic area was high and intensity was Remarkable. Fig 4 shows

Fig 2: Masson's Trichome Staining of experimental rat liver



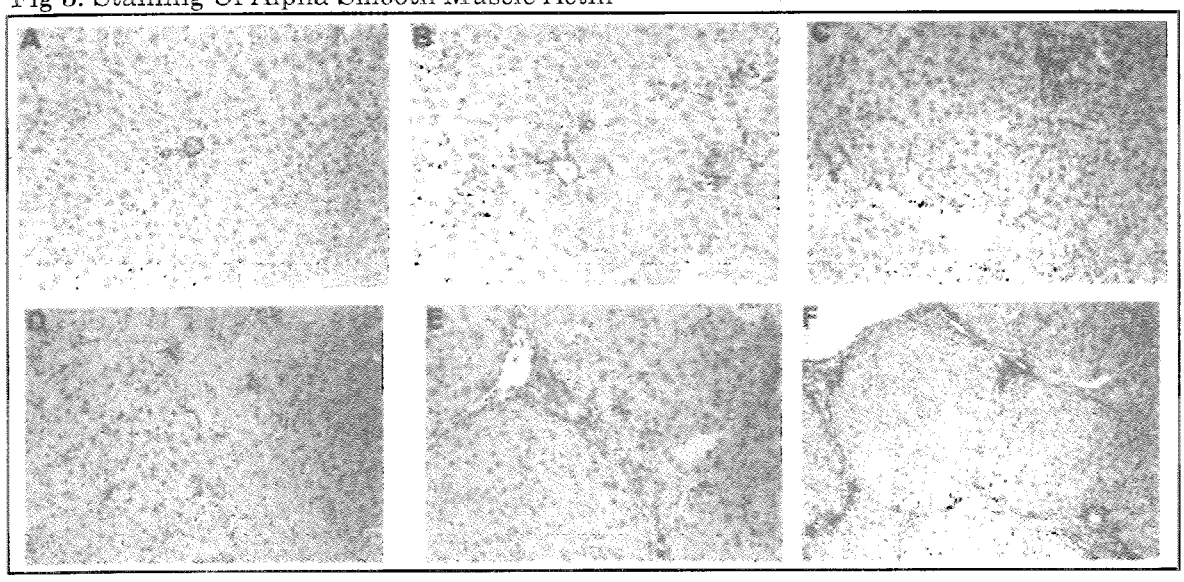
A Control Liver: Normal Architecture with central vein and radiating cords
B Day 3: Slight percentile fibrosis
C Day 5: Bridging fibrosis with initiation of collagen fibers
D Day 7: Liver specimen demonstrating well developed fibrosis
E Day 14: Focal fibrosis and clear cirrhosis
F Day 21: Delineated cirrhosis

Immunohistochemical staining of alpha smooth muscle actin demonstrating activated hepatic stellate cells during DMN induced experimental liver fibrosis in rats and effect of administration of Premna Tomentosa on activation of stellate cells. Control Liver shows absence of α - smooth muscle actin staining, Liver after Premna tomentosa treatment shows absence of α-smooth muscle actin Staining and DMN induced liver showing activation of hepatic stellate cells and Necrosis. DMN + Premna tomentosa shows absence of activated stellate cells, DMN (Day 14) Abundant number of activated stellate cells and early fibrosis, DMN + Premna Tomentosa (Day 14) Activation of stellate cells and beginning of necrosis, DMN (Day 21) marked staining of activated stellate cells in well-developed fibrotic zones. DMN + Premna Tomentosa (Day 21) Activated stellate cells and early necrosis.

DISCUSSION

DMN induced liver fibrosis in rat is a well-established, reproducible model and has several similarities with human liver cirrhosis (9). In our present study preventive role of Premna tomentosa was studied in DMN induced hepatic fibrosis in rats. Increased deposition of collagen and hyaluronic acid plays a prominent role in the pathogensis of liver fibrosis during DMN induction. Treatment with Premna tomentosa reduced both levels. Increased deposition of collagen and hyaluronic acid was found during induction with DMN and treatment with Premna tomentosa reduced both collagen as well as hyaluronic acid deposition. Lipid peroxidation was found in group - 2 during DMN induction, treatment with Premna tomentosa decreased the MDA levels, which shows the free radical scavenging ability by Premna tomentosa. α-SMA was stained a marker for activated stellate cells. Induction with DMN increased the activated stellate cells. While

Fig 3: Staining Of Alpha Smooth Muscle Actin

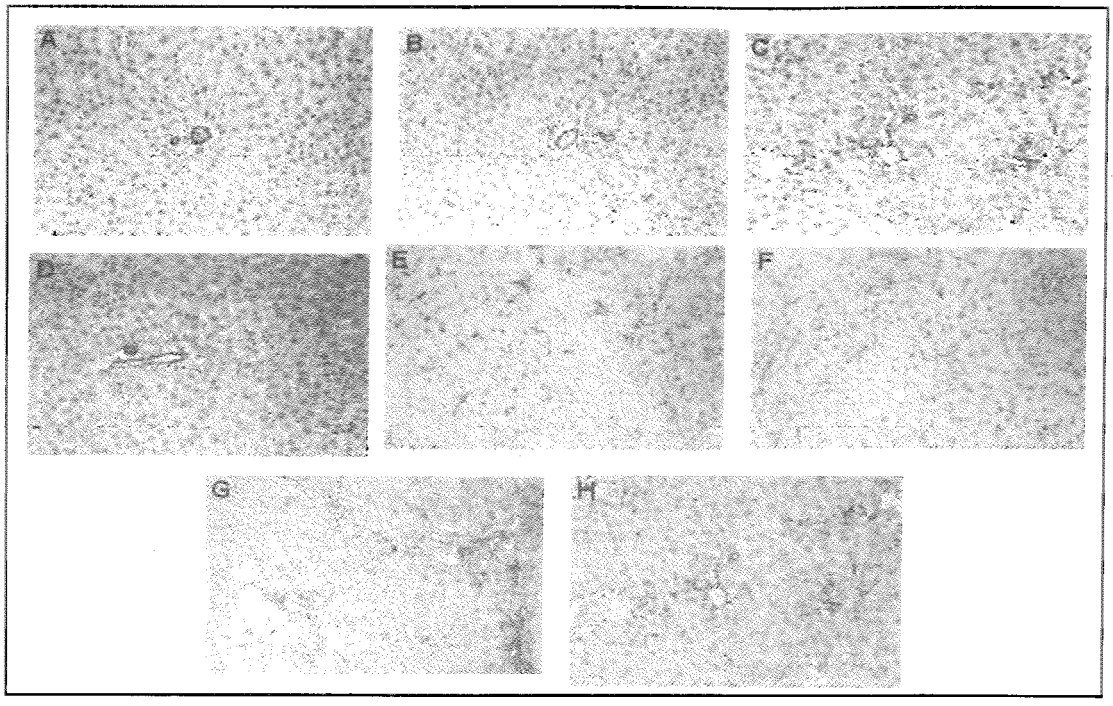


A Control Liver: Staining of alpha smooth muscle actin absent totally.
B Day 3: Prominent number of stellate cells become activated and stained for alpha smooth muscle actin
C Day 5: The number of alpha smooth muscle actin stained stellate cells increased
D Day 7: Density of positive stained stellate cells in necrotic zone was high
E Day 14: Staining was restricted to fibrotic zone
F Day 21: Density of positive stained cells in necrotic area was high and intensity Was Remarkable

treatment with Premna tomentosa decreased the activation of stellate cells. Hepatic fibrosis, characterized by an increased production and deposition of extracellular matrix component accompanies most chronic liver disorders and its presence is a major factor contributing to hepatic failure (21, 22). Although the mechanism of liver fibrosis is not fully understood, activated hepatic stellate cells play an important role in connective tissue synthesis and deposition during fibrogenesis (23).

Determination of membrane associated enzyme activities like ATPases indicate changes in membrane under pathological conditions (24). ATPase is lipid dependent, membrane - bound enzymes involved in active transport process and have been indicated in the pathogenesis of liver cell injury (25). Enhanced susceptibility of membrane to lipid peroxides can lead to loss of protein thiol, thereby changing membrane functions (26). Further, toxic insult of liver can promote a variety of chemical reactions including depletion of GSH, which affect membrane-bound ATPases (4). Decreased activities of Na^+ K^+ ATPase and Ca^{++} ATPase were observed in group 2 DMN induced rats. Decreased activity of Na^+ K^+ ATPase can lead to a decrease in sodium efflux and thereby alter the membrane permeability (27). Premna tomentosa extract pre-treatment in group 4 rats restored the levels of Na^+ K^+ ATPase to near normal which may be due to the antioxidant property of the extract (12) which arrests the free radical-induced damage. Hepatocellular damage by a wide variety of toxins may be mediated via the disruption of calcium homeostasis (28). This may involve exchange of calcium between the intracellular pools and/or the inhibition of efflux of calcium

Fig 4: Immunohistochemical staining of alpha smooth muscle actin demomstrating activated hepatic stellate cells during DMN induced experimental liver fibrosis in rats and effect of administration of Premna Tomentosa on activation of stellate cells



A - Control Liver shows absence of a - smooth muscle actin staining
B - Liver after Premna tomentosa treatment shows absence of a - smooth muscle actin Staining
C - DMN induced liver showing activation of hepatic stellate cells and Necrosis
D - DMN + Premna tomentosa shows absence of activated stellate cells
E - DMN (Day 14) Abundant number of activated stellate cells and early fibrosis
DMN + Premna Tomentosa (Day 14) Activation of stellate cells and beginning of necrosis.
G - DMN (Day 21) marked staining of activated stellate cells in well developed fibrotic zones.
H - DMN + Premna Tomentosa (Day 21) Activated stellate cells and early necrosis.

through the plasma membrane. Ca^{++} ATPase (29). The inhibition of these transport systems in the cells may result in a sustained increase in cytosolic Ca^{++} concentrations producing over stimulation of cellular processes leading ultimately to cell death (30).

Premna tomentosa shows inhibitory effect against the activation of liver stellate cells during DMN induced liver fibrosis in rats. The rate of activation of liver stellate cells was monitered by immunohistochemical staining of a SMA as a marker for activated stellate cells and the administration of drug significantly reduced activated liver stellate cells. It is reported that Premna tomentosa inhibits metal induced oxidative damage in cultured hepatocytes and hepatic lysosomal fractions.

The study results showed the treatment with Premna tomentosa had a protective role during DMN induction. Hence these results show antifibrotic nature of Premna tomentosa

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